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14. ABSTRACT

Mammographic breast density is one of the strongest known risk factors for breast cancer, and a marker of cancer risk for both breasts. Women with dense tissue in more than 75% of the breast have been shown to be at a 4-5 fold increased risk of breast cancer compared to women who have mostly fatty breasts. Greater breast density also affects mammographic sensitivity for breast cancer; mammographic sensitivity declines significantly with greater breast density. Information on the etiology of breast density is currently limited. Identifying factors that affect breast density and understanding the underlying mechanisms may help reduce breast cancer risk and improve early detection of breast cancer, thereby reducing morbidity and mortality. Increasing evidence suggests that inflammatory cytokines may influence breast cancer risk. To gain further insight into the role of inflammatory cytokines in the etiology of breast density, we investigated associations between circulating cytokine levels, common genetic variation in cytokine genes, and mammographic breast density using existing data and banked specimens from the Mammograms and Masses Study (MAMS). This final report summarizes the work conducted over the entire research period.

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Breast cancer; breast density; cytokines; genetic variation

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INTRODUCTION

Mammographic breast density is one of the strongest known risk factors for breast cancer, and a marker of cancer risk for both breasts [1, 2]. Women with dense tissue in more than 75% of the breast have been shown to be at a 4-5 fold increased risk of breast cancer compared to women who have mostly fatty breasts [1]. Early detection of breast cancer is critical for reducing mortality. Unfortunately, greater breast density also negatively affects mammographic sensitivity for breast cancer; the sensitivity declines significantly with greater breast density. Information on the etiology of breast density is currently limited, and the biological mechanism by which mammographic density is associated with breast cancer risk is unclear. Identifying factors that affect breast density and understanding the underlying mechanisms may help reduce breast cancer risk and also improve early detection of breast cancer, thereby reducing morbidity and mortality. Various evidence suggest that exposure to sex hormones, estrogens in particular, may be an important factor in breast density. Changes in density have been observed in response to hormone replacement therapy use and use of tamoxifen (3, 4). Inflammatory cytokines, specifically tumor necrosis factor (TNF)-α and interleukin (IL)-6, have emerged as critical regulators of estrogen synthesis in breast tissues [5], and may so affect breast density and breast cancer risk. In line with this, polymorphisms in *IL6* have been found associated with breast cancer risk and to modify the association between estrogen and aspirin and breast cancer risk [6]. Moreover, we recently observed lower circulating estradiol levels among postmenopausal women reporting NSAID use [7]. To gain further insight into the role of inflammatory cytokines in the etiology of breast density, this study investigated associations between circulating cytokine levels, genetic variation in cytokine genes, and breast density. Existing data and banked specimens from women who participated in a recently completed, cross-sectional study on hormones and breast density, the Mammograms and Masses Study (MAMS), were used.

BODY

For this study, we used existing data and banked specimens from women who participated in a previously completed, cross-sectional study on hormones and breast cancer, the MAMS study. The University of Pittsburgh Institutional Review Board reviewed and approved the MAMS study protocol, and all study participants provided written informed consent. We received approval from USAMRMC for the use of human anatomical substances.

A study specific database was created which in addition to relevant questionnaire and clinical data (downloaded from the MAMS database) contained information on the evaluated cytokine levels and genotypes. Data checks were conducted to ensure correctness of the data.

A. CYTOKINE LEVELS AND MAMMOGRAPHIC DENSITY

Circulating IL-6, TNF-α and CRP levels and mammographic density:

Study population

Study participants were selected from the MAMS study population. The MAMS study population consists of 1,133 pre- and postmenopausal women (18 years and older) from the Greater Pittsburgh area [7,8]. For this study, we excluded women who had been diagnosed with breast cancer (*N*=264), and subsequently excluded all women who were not postmenopausal

(*N*=222), had no available mammogram data (*N*=53), did not complete the questionnaire (*N*=26), reported a prior history of cancer after enrollment into MAMS (*N*=9), had no available plasma sample (*N*=8) or whose blood draw was more than 180 days from their mammogram date (*N*=9), leaving a final total of 145 benign controls (women with benign breast disease, not breast cancer) and 397 well controls. Premenopausal women were excluded from the analyses because both mammographic density and cytokine levels vary during the menstrual cycle due to fluctuating hormone levels [9,10] and specific information on day of the menstrual cycle at time of mammogram was not available for the study participants.

The total number of individuals used to evaluate associations between cytokine levels and mammographic density was lower than proposed mainly due to excluding pre-menopausal women from the analysis (see above) and missing information on breast density.

Cytokine level determination

Circulating levels of IL-6, TNF-α, and CRP were measured in frozen stored EDTA plasma samples by the Laboratory for Clinical Biochemistry Research (LCBR) at the University of Vermont (Colchester, VT; Dr. Russell Tracy's laboratory). Samples were shipped to the LCBR packed in dry ice using overnight courier service. Investigators at the LCBR were blinded to the identity, demographic and risk factor characteristics, and mammographic density status of the samples. To evaluate assay reproducibility, masked, duplicate samples (6.6% of total study samples) were randomly distributed throughout the batch of samples. Plasma IL-6 levels were measured using a high sensitivity enzyme-linked immunosorbent assay (ELISA; Human IL-6 Quantikine® HS, HS600B) from R&D Systems (Minneapolis, MN). The detectable limit for IL-6 was 0.10 pg/ml, and the average coefficient of variation (CV) was 16.0%. TNF-α levels were measured by a singleplex immunoassay using Luminex technology (Human Cytokine LINCOplex Kit Singleplex TNF-α, HCYTO-60K-1TNFA; Linco Research, Inc., St. Charles, MO). This assay can measure TNF-α concentrations ≤3.2 pg/ml, and the average CV was 10.8%. CRP levels were measured using the BNII nephelometer from Dade Behring utilizing a particle-enhanced immunonepholometric assay. This assay has a detection limit of 0.16 μg/ml, and the average CV was 9.6%.

Mammographic density measurements

Copies of participants' most recent screening mammograms were obtained with their permission. The assessment of mammographic measures has been described in detail in Reeves *et al*. [8]. Briefly, one expert reader read all mammograms, which were copies of the original films. This reader was masked to the identity, status, and demographic and risk factor characteristics of the subject. Total breast area and all dense regions were measured using a compensating polar planimeter (LASICO) on the craniocaudal view with the side of breast (right or left) randomly chosen for each participant. Dense breast area is the sum of all dense regions; nondense breast area was calculated by subtracting dense breast area from total breast area; percent density was calculated by dividing dense breast area by total breast area and multiplying that by 100.

Statistical analysis

All analyses were performed separately for benign controls and well controls. Differences between benign and well controls were assessed using chi-square tests for categorical variables

and *t*-tests or analysis of variance (ANOVA) for continuous variables. The normality of the distribution of circulating IL-6, TNF-α, and CRP levels, and dense breast area, nondense breast area, and percent density was assessed graphically using quantile–quantile plots. To improve normality, natural log transformations were applied to the inflammatory markers and square root transformations were applied to the mammographic density measures. Pearson's correlation coefficient was used to examine the correlation between cytokine levels and mammographic measures; Fisher's z transformations were used to test differences between the correlation coefficients. Linear regression was used to further assess the association between each inflammatory marker and mammographic density. The assumptions needed for linear regression were met. Unadjusted, age-adjusted, age- and BMI-adjusted, and multivariable-adjusted regression models were run for each combination of inflammatory marker and mammographic density measure. The multivariable model included covariates found to be associated with mammographic density and/or breast cancer in previous studies: age (continuous), BMI (<25 kg/m^2, 25 to <30 kg/m^2, ≥30 kg/m^2), race (white, other), smoking (never, former, current), current NSAID use (nonuser, user), first-degree relative with breast cancer (no, yes), age at menarche (≤12, >12), age at menopause (<50, ≥50), type of menopause (natural, hysterectomy without oophorectomy, hysterectomy with uni- or bilateral oophorectomy), prior breast biopsy (no, yes), ever been pregnant (no, yes), and postmenopausal hormone therapy use status (never, former, current). We subsequently repeated the regressions stratified by BMI, current NSAID use, and time between blood draw and mammogram. *P* values <0.05 were considered statistically significant. Analyses were performed using Stata (version 10.0; Stata Corporation, College Station, TX) and SAS (version 9.2; SAS Institute Inc., Cary, NC) software.

Results

Characteristics of the study population by control status are presented in Table 1. Mean age among benign controls was statistically significantly lower than among well controls. Additionally, benign controls reported having had a prior breast biopsy, to be older than 12 years of age at menarche, to have gone through menopause before age 50, and to be current users of postmenopausal hormone therapy statistically significantly more often than well controls. Current use of NSAIDs was significantly more common among the well controls.

The distributions of the inflammatory markers and the mammographic density measures by control status are shown in Table 2. The age-adjusted geometric mean of CRP was statistically significantly higher among benign controls (2.07 μg/mL) than among well controls (1.63 μg/mL; p=0.02). No significant differences between benign controls and well controls were observed for IL-6 and TNF-α. Regarding the mammographic density measures, age-adjusted mean dense breast area (42.8 cm^2 vs. 36.1 cm^2; p=0.02) and age-adjusted mean percent density (31.2% vs. 26.0%; p=0.01) were both statistically significantly higher among benign controls than among well controls. Age-adjusted mean nondense breast area did not differ significantly between the two groups (97.8 cm^2 vs. 108.1 cm^2; p=13).

No significant correlations were observed between circulating IL-6, TNF-α and CRP levels and dense breast area among benign or well controls (Table 3). However, all three cytokines were statistically significantly, positively correlated with nondense breast area in both control groups (benign controls: IL-6: ρ=0.32, p<0.001; TNF-α: ρ=0.22, p<0.001; CRP: ρ=0.25, p=0.003; well controls: IL-6: ρ=0.30, p<0.001; TNF-α: ρ=0.26, p<0.001; CRP: ρ=0.36, p<0.001), and statistically significantly, negatively correlated with percent density among well

controls (IL-6: ρ=-0.20, p<0.001; TNF-α: ρ=-0.18, p<0.001; CRP: ρ=-0.23, p<0.001). Among benign controls the correlation with percent density was statistically significant for IL-6 (ρ=-0.21, p=0.01) and borderline significant for CRP (ρ=-0.16, p=0.05). TNF-α levels were also negatively correlated with percent density among benign controls, yet this association was not statistically significant. No statistically significant differences in correlation coefficients for IL-6, TNF-α, and CRP were observed between benign controls and well controls (Table 3).

Results from the age-adjusted, age- and BMI-adjusted, and multivariable-adjusted linear regression models are presented in Table 4. For both benign controls and well controls, no statistically significant associations were observed between levels of IL-6, TNF-α and CRP and dense breast area in any of the models, but all three inflammatory markers were statistically significantly associated with nondense breast area in the age-only adjusted model (benign controls: IL-6: β=1.44, p<0.001; TNF-α: β=1.46, p=0.007; CRP: β=0.70, p=0.003; well controls: IL-6: β=1.49, p<0.001; TNF-α: β=1.78, p<0.001; CRP: β=1.18, p<0.001). The associations with nondense breast area became non-significant after additional adjustment for BMI and other variables. Among benign controls, IL-6 was statistically significantly associated with percent density in the age-only-adjusted model (β=-0.55, p=0.02). This association became non-significant upon further adjustment for BMI, and remained non-significant upon adjustment for additional covariates. No significant associations were observed for TNF-α and CRP levels with percent density among women with benign breast disease in any model. Among well controls, all three inflammatory markers were statistically significantly associated with percent density in the age-only-adjusted model (IL-6: β=-0.54, p<0.001; TNF-α: β=-0.71, p<0.001; CRP: β=-0.43, p<0.001). These associations became non-significant after additional adjustment for BMI and other variables. Subsequently, regressions were repeated stratified by BMI, current NSAID use, and time between blood draw and mammogram; results were generally similar to those observed overall (data not shown).

Table 1: Selected characteristics of the study population by control status (N_{total}=542)

	Benign controls (N=145)[a] N (%)	**Well controls** (N=397)[a] N (%)	*P*[b]
Age (years; mean ± SD)	58.3 ± 7.4	62.0 ± 8.1	<0.001
Age (years)			<0.001
Younger than 50	12 (8.3)	4 (1.0)	
50-59	68 (46.9)	180 (45.3)	
60-69	55 (37.9)	135 (34.0)	
70 or older	10 (6.9)	78 (19.7)	
Race: White	136 (93.8)	373 (94.0)	0.94
Body mass index (kg/m^2; mean ± SD)	27.9 ± 5.9	28.3 ± 6.0	0.57
Body mass index (kg/m^2)			0.46
Normal (less than 25.0)	44 (30.6)	131 (33.0)	
Overweight (25.0-<30.0)	58 (40.3)	137 (34.5)	
Obese (30.0 or more)	42 (29.2)	129 (32.5)	
Smoking status			0.07
Never	81 (53.6)	227 (57.2)	
Former	45 (31.3)	144 (36.3)	
Current	18 (12.5)	26 (6.6)	
Prior breast biopsy	60 (41.7)	57 (14.4)	<0.001
First-degree relative with breast cancer	18 (12.5)	56 (14.2)	0.61
Age at menarche (years)			0.04
12 or younger	58 (40.0)	197 (49.8)	
Older than 12	87 (60.0)	199 (50.3)	
Ever been pregnant	121 (83.5)	332 (83.6)	0.96
Age at first pregnancy lasting ≥6 months			0.36
Never pregnant/no pregnancies ≥6 months	32 (22.2)	80 (20.2)	
Younger than 20	18 (12.5)	35 (8.8)	
20-24	52 (36.1)	143 (36.0)	
25-29	27 (18.8)	89 (22.4)	
30 or older	15 (10.4)	50 (12.6)	

Table 1 cont.	**Benign controls** (N=145)[a] N (%)	**Well controls** (N=397)[a] N (%)	*P*[b]
Age at menopause (years)			<0.001
Younger than 50	85 (59.9)	164 (42.2)	
50 or older	57 (40.1)	225 (57.8)	
Type of menopause			0.13
Natural menopause	90 (65.7)	275 (72.4)	
Hysterectomy without oophorectomy	16 (11.7)	48 (12.6)	
Hysterectomy with uni- or bilateral oophorectomy	31 (22.6)	57 (15.0)	
Postmenopausal hormone therapy use			<0.001
Never	27 (18.8)	140 (35.3)	
Former	43 (29.9)	203 (51.1)	
Current (within previous 3 months)	74 (51.4)	54 (13.6)	
Current NSAID use	42 (34.4)	194 (49.2)	0.004

[a]The numbers do not always add up to the total number of benign and well controls due to missing information.
[b] Chi-square tests for categorical variables and *t*-tests for continuous variables.

Table 2: Distribution of inflammatory markers and mammographic density measures by control status

	Benign controls				Well controls				
	N	Mean (SD)	Median	Age-adjusted transformed mean[a]	N	Mean (SD)	Median	Age-adjusted transformed mean[a]	*P*[b]
Inflammatory markers[c]									
IL-6 (pg/ml)	145	2.67 (2.72)	1.97	2.12	397	2.89 (2.91)	1.98	2.17	0.71
TNF-α (pg/ml)	145	3.00 (1.60)	2.59	2.67	394	2.99 (1.83)	2.68	2.63	0.70
CRP (μg/ml)	142	4.16 (8.57)	2.17	2.07	381	2.92 (4.17)	1.47	1.63	0.02
Mammographic density measures									
Dense breast area (cm^2)	145	48.0 (30.6)	44.6	42.8	397	40.9 (26.6)	36.7	36.1	0.02
Nondense breast area (cm^2)	145	106.0 (71.9)	90.5	97.8	397	120.7 (76.3)	100.1	108.1	0.13
Percent density (%)	145	35.2 (18.8)	34.2	31.2	397	29.6 (19.4)	27.5	26.0	0.01

[a] Transformed mean is geometric mean for the inflammatory markers. For the mammographic density variables, the transformed mean is a mean calculated on the square root scale that was subsequently transformed back to the original scale.
[b] *P* values from ANOVA, comparing distributions among benign controls to well controls using natural log transformations of the inflammatory markers and square root transformations of the mammographic density variables with adjustment for age.
[c] TNF-α levels could not be measured for 3 well controls; CRP levels could not be measured for 3 benign controls and 16 well controls

Table 3: Correlations between inflammatory markers and mammographic density measures by control status[a]

	Benign controls			Well controls			
	N	ρ	*P*	N	ρ	*P*	P^b
Dense breast area							
IL-6	145	-0.03	0.72	397	-0.06	0.24	0.77
TNF-α	145	0.04	0.64	394	-0.01	0.78	0.59
CRP	142	-0.01	0.95	381	-0.03	0.60	0.83
Nondense breast area							
IL-6	145	0.32	<0.001	397	0.30	<0.001	0.90
TNF-α	145	0.22	<0.001	394	0.26	<0.001	0.65
CRP	142	0.25	0.003	381	0.36	<0.001	0.22
Percent density							
IL-6	145	-0.21	0.01	397	-0.20	<0.001	0.90
TNF-α	145	-0.11	0.19	394	-0.18	<0.001	0.44
CRP	142	-0.16	0.05	381	-0.23	<0.001	0.50

[a] Calculated using Pearson's correlation coefficient with natural log transformations of the inflammatory markers and square root transformations of the mammographic density variables.
[b] *P* values for comparison of correlation coefficients between benign controls and well controls

Table 4: Results of linear regressions of mammographic density measures on inflammatory markers by control status[a]

Benign controls									
		Age-adjusted			Age- and BMI-adjusted			Fully adjusted[b]	
	N	β (SE)	*P*	N	β (SE)	*P*	N	β (SE)	*P*
Dense breast area									
IL-6	145	-0.13 (0.28)	0.64	144	-0.07 (0.31)	0.83	111	-0.07 (0.39)	0.85
TNF-α	145	0.18 (0.37)	0.63	144	0.26 (0.39)	0.50	111	0.40 (0.50)	0.43
CRP	142	-0.01 (0.16)	0.95	141	0.001 (0.17)	0.99	109	0.16 (0.21)	0.45
Nondense breast area									
IL-6	145	1.44 (0.39)	<0.001	144	0.23 (0.35)	0.52	111	0.09 (0.45)	0.84
TNF-α	145	1.46 (0.53)	0.007	144	0.50 (0.44)	0.26	111	0.03 (0.58)	0.96
CRP	142	0.70 (0.23)	0.003	141	0.16 (0.19)	0.42	109	0.06 (0.24)	0.82
Percent density									
IL-6	145	-0.55 (0.23)	0.02	144	-0.08 (0.23)	0.74	111	-0.03 (0.30)	0.93
TNF-α	145	-0.42 (0.31)	0.18	144	-0.03 (0.29)	0.92	111	0.19 (0.35)	0.62
CRP	142	-0.26 (0.13)	0.06	141	-0.05 (0.13)	0.69	109	0.06 (0.17)	0.71
Well controls									
		Age-adjusted			Age- and BMI-adjusted			Fully adjusted[†]	
	N	β (SE)	*P*	N	β (SE)	*P*	N	β (SE)	*P*
Dense breast area									
IL-6	397	-0.16 (0.16)	0.32	397	-0.07 (0.17)	0.69	368	-0.15 (0.18)	0.41
TNF-α	394	-0.04 (0.23)	0.87	394	0.09 (0.24)	0.71	365	0.04 (0.25)	0.87
CRP	381	-0.06 (0.11)	0.57	381	0.01 (0.12)	0.96	353	0.02 (0.13)	0.85
Nondense breast area									
IL-6	397	1.49 (0.24)	<0.001	397	0.39 (0.20)	0.05	368	0.34 (0.22)	0.12
TNF-α	394	1.78 (0.34)	<0.001	394	0.35 (0.28)	0.21	365	0.32 (0.30)	0.29
CRP	381	1.18 (0.16)	<0.001	381	0.27 (0.14)	0.06	353	0.29 (0.16)	0.06

Table 4: (continued)

Percent density									
IL-6	397	-0.54 (0.14)	<0.001	397	-0.11 (0.14)	0.43	368	-0.13 (0.15)	0.38
TNF-α	394	-0.71 (0.20)	<0.001	394	-0.16 (0.19)	0.39	365	-0.17 (0.20)	0.41
CRP	381	-0.43 (0.09)	<0.001	381	-0.08 (0.10)	0.44	353	-0.07 (0.10)	0.48

[a]Regressions performed using natural log transformations of the inflammatory markers and square root transformations of the mammographic measures

[b]Adjusted for age, race, BMI, smoking, current NSAID use, first-degree relative with breast cancer, age at menarche, age at menopause, type of menopause, prior breast biopsy, ever been pregnant, and postmenopausal hormone therapy use.

Circulating sTNFR1 and sTNFR2 levels and mammographic density:

Study population

Study participants were selected from the MAMS study population. The MAMS study population consists of 1,133 pre- and postmenopausal women (18 years and older) from the Greater Pittsburgh area [7,8]. For this study, we excluded women who had been diagnosed with breast cancer (*N*=264), were not postmenopausal (*N*=222), used hormone therapy at time of enrollment (*N*=128), had no available mammogram data (*N*=53), did not complete the questionnaire (*N*=26), reported a prior history of cancer after enrollment into MAMS (*N*=9), had no available plasma sample (*N*=8), whose blood draw was more than 180 days from their mammogram date (*N*=9), or had no available soluble tumor necrosis factor receptor (sTNFR) levels results (*N*=38) leaving a final total of 376 women. Premenopausal women were excluded from the analyses because both mammographic density and cytokine levels vary during the menstrual cycle due to fluctuating hormone levels [9,10] and specific information on day of the menstrual cycle at time of mammogram was not available for the study participants. We excluded women who used hormone therapy at time of enrollment because hormone therapy use is associated with increases in mammographic breast density [3].

Cytokine level determination

Circulating levels of sTNFR1 and sTNFR2 were measured in frozen stored EDTA plasma samples by the LCBR at the University of Vermont (Colchester, VT; Dr. Russell Tracy's laboratory) using commercially available sTNFR1 and sTNFR2 antibody bead kits for human plasma (BioSource International, Camarillo, CA, USA). Samples were shipped to the LCBR packed in dry ice using overnight courier service. Investigators at the LCBR were blinded to the identity, demographic and risk factor characteristics, and mammographic density status of the samples. The standard curve ranges were 23,400 to 30 pg/mL and 11,400 to 20 pg/mL for sTNFR1 and sTNFR2, respectively. Using control plasma, the laboratory reported within-assay coefficients of variation of 17.5% and 17.4% for sTNFR1 and sTNFR2 concentrations, respectively. The inter-assay coefficients of variation calculated from the analytic results for 40 masked duplicate plasma samples were 30.0% and 22.4% for sTNFR1 and sTNFR2 concentrations, respectively

Mammographic density measurements
See above

Statistical analysis
Descriptive statistics for baseline characteristics were calculated (mean plus standard deviation [SD] for continuous variables; frequency for categorical variables). Baseline characteristics were compared across quartiles of percent mammographic density, and sTNFR1 and sTNFR2 levels using ANOVA for continuous and chi-square tests or Fisher's exact tests for categorical variables. Pearson's correlation coefficient was used to examine the correlation between sTNFR levels and percent mammographic density. Fisher's z transformation was used to estimate 95% confidence limits for the correlation coefficients. Percent mammographic density was the primary focus of this study. However, associations between sTNFR levels and dense breast area and nondense breast area are reported as well. Age (in years), BMI (kg/m^2), prior breast biopsy (yes/no), former hormone therapy use (yes/no), current alcohol use (yes/no), age at first birth (<30 vs. ≥30 or nulliparous), education (high school graduate vs. any post-secondary education), aspirin or other anti-inflammatory drug use within 48 hrs of blood draw (yes/no), age at menopause (continuous), years since menopause (continuous), number of live births (0, 1, 2, 3, 4, or 5+; continuous), and nulliparity (yes/no) were all evaluated as potential confounding variables due to their known associations with breast density, breast cancer, or sTNFR levels, or evidence of a difference in the covariate across quartiles of percent breast density or sTNFR levels (P<0.10). We used forward, stepwise multivariable linear regression to develop a model describing the factors associated with percent breast density (excluding sTNFR1 and sTNFR2). The model building process proceeded as follows: First, we separately regressed on the outcome variable (percent mammographic density) each potential confounding variable. The variable that explained the largest proportion of the variation in percent breast density (R^2) was then selected as the first variable to be entered into the regression equation. Each remaining explanatory variable was then regressed on percent breast density jointly with the first variable, and partial F statistics were determined. The variable with the largest partial F statistic (providing the largest gain in explanatory power) was then added as the second variable in the multiple regression equation (P-value to enter model=0.10), and this process was repeated for the remaining variables until the final model was reached (e.g., the test for the partial F statistic was not significant for the variables not yet in the model). Age-adjusted, age- and BMI-adjusted, and multivariable-adjusted regression models (covariates determined to explain the largest proportion of variation in percent breast density in the stepwise linear regression process described above) were run to assess the relation of the sTNFR levels and percent breast density. To improve normality, natural log transformations were applied to the sTNFR1 levels; mammographic density and sTNFR2 levels were normally distributed and analyses were conducted with these variables in the natural scale. Bonferroni correction was used to control for Type I error across the 2 cytokine comparisons; a P-value of less than 0.025 (0.05/2) was required for statistical significance. Analyses were performed using Stata (version 10.0; Stata Corporation, College Station, TX) and SAS (version 9.2; SAS Institute Inc., Cary, NC) software.

Results
Characteristics of the study population are presented in Table 5. Mean age was 62 years, ranging from 42-85. The majority of the population was Caucasian (94%) and attended post-secondary education (75%). Mean years since menopause was 14, ranging from 1-43, while mean percent

breast density was 29.7% , ranging from 0-94.9%. sTNFR1 and sTNFR2 levels were positively correlated with one another (r=0.49, *P*<0.0001; not in Table). One hundred sixty three (44%) participants reported taking aspirin or another anti-inflammatory agent within 48 hours of blood draw (not in Table).

Table 6 shows the characteristics of the study population by quartiles of percent mammographic density. Women with higher percent mammographic density were younger, and thus had fewer years since menopause; had a lower BMI; were more likely to have attended post-secondary education, to report current consumption of alcohol, to be nulliparous and/or have a later age at first birth, to be former hormone therapy users, and prior breast biopsy; and were less likely to have taken aspirin or another anti-inflammatory agent at blood draw than women with lower percent mammographic density.

We also evaluated characteristics of the study population by quartiles of sTNFR1 level and sTNF2 level. Women with higher sTNFR1 and sTNFR2 levels had a higher BMI and larger nondense breast area than women with lower sTNFR levels. Women with higher sTNFR2 levels were older, had a greater number of years since menopause and were less educated than women with lower sTNFR2 levels (data not shown).

Before adjustment, both sTNFR1 and sTNFR2 were significantly inversely correlated with percent mammographic density (r = -0.14, *P*=0.007, and r = -0.13, *P*=0.01, respectively; Table 7). As expected, an opposite relationship was observed for nondense breast area, which was positively correlated with sTNFR1 and sTNFR2 levels (Table 8). sTNFR levels were not associated with the dense area of the breast (r= -0.05, p=0.31 and r = -0.02, *P*=0.65) (Table 9).

Results of the multivariable linear regression analyses are shown in Tables 10-12. The inverse associations observed between the sTNFR levels and percent mammographic density remained after adjustment for age (Table 10); however, age and each sTNFR explained only 3% of the variation in percent mammographic density (R^2=0.03). After adjustment for BMI, 24% of the variation in percent mammographic density was explained, but the inverse association between the sTNFR levels and percent mammographic density was no longer statistically significant. The covariates associated with percent mammographic density in the stepwise model were: age, BMI, prior breast biopsy, nulliparity and current alcohol consumption. Addition of these covariates did not further influence the relationship between the sTNFR levels and percent mammographic density, and increased the R^2 to 28%. No association was observed between the sTNFR levels and dense breast area (Table 12), while positive age-adjusted associations resulted between the sTNFR levels and nondense breast area (Table 11). Again, this association was no longer significant after adjustment for BMI.

Table 5: Characteristics of the study population (Ntotal=376)

	N (%)
Age (in years; mean ± SD)	62 ± 8
Race: White	353 (94)
Education level:> High school	274 (75)
Body mass index (in kg/m^2; mean ± SD)	28.4 ± 6.1
Cigarette Smoking	
Never	219 (58)
Former	133 (35)
Current	23 (6)
Current alcohol consumption ≥1/week for ≥6 months (%)	
No	263 (72)
Yes: <12g/day	68 (19)
Yes: ≥12g/day	35 (9)
Age at menarche (in years)	
<12	72 (19)
12-13	223 (59)
≥14	80 (21)
Nulliparous: no	295 (78)
Parity	
Nulliparous	81 (21)
1	43 (11)
2	113 (30)
3	79 (21)
4	32 (9)
5+	28 (7)
Age at first birth: <30	249 (66)
Age at menopause (in years; mean ± SD)	48 ± 5
Years since menopause (mean ± SD)	14 ± 10
Hormone therapy use: past use	225 (60)
Family history of breast cancer: yes	54 (14)
Prior breast biopsy: yes	70 (19)
Percent density (mean ± SD)	29.7 ± 19.5
Dense breast area (cm^2; mean ± SD)	40.9 ± 26.2
Nondense breast area (cm^2; mean ± SD)	121.5 ± 78.0
sTNFR1 (pg/ml; mean ± SD)	2794.7 ± 2314.9
sTNFR2 (pg/ml; mean ± SD)	2662.6 ± 1202.8

Table 6: Characteristics of the study population by percent mammographic density (in quartiles; Q)

	Mammographic density (%)				
	Q1 (0-14.0)	**Q2 (14.1-27.4)**	**Q3 (27.5-42.0)**	**Q4 (42.2-94.9)**	***P*-value**
Median % density	8.6	20.4	34.1	56.6	
Median sTNFR1 pg/mL	2612.9	2522.7	2188.9	1961.5	
Median sTNFR2 pg/mL	2541.0	2680.6	2497.5	2433.9	
Mean (SD)					
Age (in years)	62 (8)	63 (8)	63 (8)	60 (8)	0.03
Body mass index (in kg/m^2)	32.0 (6.1)	29.7 (5.9)	27.2 (5.1)	24.7 (4.6)	<0.0001
Age at menopause (in years)	48 (5)	48 (5)	48 (6)	48 (5)	0.83
Years since menopause	14 (9)	15 (10)	15 (10)	11 (9)	0.05
Dense breast area (in cm^2)	15.8 (10.9)	36.4 (15.8)	45.9 (17.8)	65.6 (28.1)	<0.0001
Nondense breast area (in cm^2)	197.6 (76.3)	147.2 (64.8)	88.4 (36.0)	53.1 (28.8)	<0.0001
Frequency, n (%)					
White	88 (94)	86 (91)	90 (96)	89 (95)	0.65
> High school	62 (67)	63 (68)	72 (78)	77 (85)	0.02
Ever smoker	40 (43)	39 (41)	43 (46)	34 (37)	0.64
Current alcohol consumption ≥1/week for ≥6 months	15 (16)	24 (26)	31 (34)	34 (37)	0.01
Age at menarche (years)					0.68
<12	21 (22)	19 (20)	19 (20)	13 (14)	
12-13	53 (56)	52 (56)	59 (63)	59 (63)	
≥14	20 (21)	22 (24)	16 (17)	22 (23)	
Nulliparous	15 (16)	15 (16)	20 (21)	31 (33)	0.01
Age at first birth ≥30 or nulliparous	27 (29)	26 (28)	32 (34)	42 (45)	0.05
Former hormone therapy use	44 (47)	56 (60)	69 (73)	56 (60)	0.003
Family history of breast cancer †	17 (18)	13 (14)	8 (9)	16 (17)	0.25
Prior breast biopsy	7 (7)	14 (15)	22 (23)	27 (29)	0.001
Aspirin or other anti-inflammatory agent within 48 hours of blood draw	53 (56)	39 (41)	39 (42)	32 (34)	0.02

Table 7: Correlation between circulating sTNFR levels and percent mammographic density

	Pearson correlation coefficient*	95% CI	*P*-value
sTNFR1 pg/mL (N=376)	-0.14	(-0.24, -0.04)	0.007
sTNFR2 pg/mL (N=376)	-0.13	(-0.23, -0.03)	0.01

*Pearson's correlation between the continuous measure of percentage of breast density and the continuous levels of log transformed values for sTNFR1 and raw values for sTNFR2.

Table 8: Correlation between circulating sTNFR levels and nondense breast area

	Pearson correlation coefficient*	95% CI	*P*-value
sTNFR1 pg/mL (N=376)	0.20	(0.10, 0.29)	<0.0001
sTNFR2 pg/mL (N=376)	0.17	(0.07, 0.27)	0.0007

*Pearson's correlation between the continuous measure of nondense breast area and the continuous levels of log transformed values for sTNFR1 and raw values for sTNFR2.

Table 9: Correlation between circulating sTNFR levels and dense breast area

	Pearson correlation coefficient*	95% CI	*P*-value
sTNFR1 pg/mL (N=376)	-0.05	(-0.15, 0.05)	0.31
sTNFR2 pg/mL (N=376)	-0.02	(-0.12, 0.08)	0.65

*Pearson's correlation between the continuous measure of dense breast area and the continuous levels of log transformed values for sTNFR1 and raw values for sTNFR2.

Table 10:. Relationship between circulating sTNFR levels and percent mammographic density.

	β (%)	SE	*P*-value	R^2 †
sTNFR1 pg/mL				
+Age (N=376)	-3.60	1.39	0.01	0.03
+BMI (N=376)	-0.77	1.26	0.54	0.24
+MV § (N=366)	-1.00	1.23	0.42	0.28
+MV2 * (N=347)	-0.63	1.46	0.66	0.30
sTNFR2 pg/mL				
+Age (N=376)	-0.002	0.001	0.03	0.03
+BMI (N=376)	-0.0003	0.001	0.70	0.24
+MV § (N=366)	-0.0003	0.001	0.68	0.28
+MV2 * (N=347)	-0.0004	0.001	0.65	0.30

MV=multivariable

†R^2 based on regression models of continuous levels of log transformed values for sTNFR1 and raw values for sTNFR2 on the continuous measure of percentage of breast density.

§ Adjusted for the following variables: age (continuous), BMI (continuous), prior breast biopsy (yes/no), nulliparous (yes/no), and current alcohol consumption (yes/no).

* Adjusted for the following variables: age (continuous), BMI (continuous), prior breast biopsy (yes/no), nulliparous (yes/no), current alcohol consumption (yes/no), prior use of hormone therapy (yes/no), post-secondary education (yes/no), aspirin use within 48 hrs of blood draw (yes/no), laboratory technician (1/2), years since menopause (continuous), and site of enrollment (biopsy vs. screening).

Table 11: Relationship between circulating sTNFR levels and nondense breast area

	β (%)	SE	*P*-value	R^2 †
sTNFR1 pg/mL				
+Age (N=376)	21.39	5.5	0.0001	0.04
+BMI (N=376)	4.54	4.02	0.26	0.51
sTNFR2 pg/mL				
+Age (N=376)	0.01	0.003	0.001	0.03
+BMI (N=376)	0.001	0.002	0.54	0.51

†R^2 based on regression models of continuous levels of log transformed values for sTNFR1 and raw values for sTNFR2 on the continuous measure of nondense breast area.

Table 12: Relationship between circulating sTNFR levels and dense breast area.

	β (%)	SE	*P*-value	R^2 †
sTNFR1 pg/mL				
+Age (N=376)	-1.81	1.89	0.34	0.005
+BMI (N=376)	-1.45	1.94	0.45	0.007
+MV § (N=366)	-1.73	1.90	0.36	0.09
sTNFR2 pg/mL				
+Age (N=376)	-0.0003	0.001	0.78	0.003
+BMI (N=376)	-0.0001	0.001	0.94	0.006
+MV § (N=366)	-0.0003	0.001	0.82	0.09

MV=multivariable

†R^2 based on regression models of continuous levels of log transformed values for sTNFR1 and raw values for sTNFR2 on the continuous measure of dense breast area.

§ Adjusted for the following variables: age (continuous), BMI (continuous), prior breast biopsy (yes/no), nulliparous (yes/no), and post-secondary education (yes/no).

B. VARIATION IN CYTOKINE GENES AND MAMMOGRAPHIC DENSITY

Variation in *IL-6, TNF-alpha, IL-6R, IL6-ST, TNFRSF1A*, and *TNFRSF1B* and mammographic density:

Study population

To evaluate the effect of variation in cytokine genes on mammographic density we used controls participating in MAMS. Details of MAMS have been described elsewhere [7,8]. Briefly, women were eligible for MAMS if they were 18 years or older and were visiting Magee-Women's Hospital (Pittsburgh, PA) or a Magee Womancare center in the greater Pittsburgh area for one of the following: a) a breast biopsy, b) an initial surgical consultation after breast cancer diagnosis, or c) a routine screening mammogram. Women were excluded if they reported a prior cancer history other than non-melanoma skin cancer, drank more than 5 alcoholic beverages per day, or weighed less than 110 pounds or more than 300 pounds. Recruitment took place from September 2001 to May 2005. The University of Pittsburgh Institutional Review Board reviewed and approved the study protocol, and all study participants provided written informed consent. The MAMS study population consists in total of 1,133 women and includes 556 women with a negative screening mammogram. Only postmenopausal women with a negative routine screening mammogram (well controls; *N*= 444) were included in the present study. We subsequently excluded all women who had no available mammogram data (*N*=32), did not complete the questionnaire (*N*=7), were not Caucasian (*N*=25), or had no available DNA (*N*=9), leaving a final total of 371 women.

Mammographic density measurements

See above

SNP Selection and Genotyping

Study participants were genotyped for 45 single nucleotide polymorphisms (SNPs) located in or near *IL6* (9 SNPs), *IL6R* (12 SNPs), *IL6ST* (7 SNPs), *TNF-α* (1 SNP), *TNFRSF1A* (7 SNPs), or *TNFRSF1B* (9 SNPs). *IL6ST* was included because IL-6 acts by binding to IL-6R which must associate with gp130 (coded for by *IL6ST*) in order for signal transduction to occur. Putative functional SNPs were selected using public databases such as the Genome Variation Server and dbSNP, and literature search. Additionally, for each gene except *TNF-α*, tagSNPs capturing common variants in the gene region were selected using data from the International HapMap project (www.hapmap.org; CEU population) and Haploview's Tagger [11,12] [minor allele frequency (MAF)>0.05 and pairwise correlation $r^2 \geq 0.80$]. All genotyping was performed at the University of Pittsburgh Genomics and Proteomics Core Laboratories (Pittsburgh, PA). SNP rs1800629 (*TNF* -308 G/A), was assessed using TaqMan (Assay ID: C___7514879_10; Applied Biosystems, Foster City, CA). All other SNPs were genotyped using MassARRAY® iPLEX Gold (Sequenom, Inc., San Diego, CA); the SNP specific and mass extend oligonucleotides, and assays were designed using Sequenom RealSNP (www.realSNP.com) and MassARRAY Assay Design version 3.1 (Sequenom, Inc., San Diego, CA). Sample duplicates (*N*=36) were included to monitor genotyping quality; no discrepant genotypes were observed. Analyses were restricted to women with genotyping call rates of ≥90%. Two study participants were excluded based on <90% call rates, leaving a total of 369 women available for analyses.

Statistical Analysis

Descriptive statistics for baseline characteristics were calculated (frequency). Deviation from Hardy-Weinberg equilibrium was assessed with the Chi-square goodness-of-fit test. With the exception of rs2228576 in *TNFRSF1A* (*P*=0.0002) and rs653667 in *TNFRSF1B* (*P*=0.0013), all SNPs were in Hardy-Weinberg equilibrium. Percent mammographic density was the primary focus of this study. Linear regression models (Proc GLM in SAS) were used to examine the relationship between each SNP and percent density. Because the number of rare-allele homozygotes in some cases was relatively small, heterozygotes and rare-allele homozygotes were combined in the analyses (common allele homozygotes were used as the reference group). Percent density was square-root transformed to normalize the distribution. For ease of interpretation, the presented means were transformed back to the original scale. To determine if there was a linear trend with increasing variant alleles, *P* values were also calculated with a linear regression model based on the number of copies of rare alleles (0, 1, 2). All models were adjusted for age (continuous), BMI (continuous), hormone therapy use (never, past, current), NSAID use (no, yes), pregnancy for at least 6 months (no, yes), and prior breast biopsy (no, yes). All significance tests were two-sided; *P* values <0.05 were considered statistically significant. All analyses were performed with use of the SAS® statistical software package (SAS version 9.2, SAS Institute Inc., Cary, NC).

Results

In total, 369 healthy postmenopausal women were genotyped. Characteristics of the study population are presented in Table 13. Mean age was 62.1 (±8.2) years, and mean BMI was 28.1 $kg.m^2$ (±5.9). Mean percent mammographic density was 30.2 (±19.6).

Table 14 shows the results of the relationship between each investigated SNP and percent mammographic density. All models were adjusted for age, BMI, hormone therapy use, NSAID

use, pregnancy for at least 6 months and prior breast biopsy. None of the evaluated SNPs in *IL6*, *TNF-α*, *TNFRSF1A* and *TNFRSF1B* were significantly associated with percent density in our study population. However, two SNPs located in *IL6R*, rs11265608 and rs64227627, and one in *IL6-ST*, rs11574780, were statistically significantly associated with percent density. For both rs11265608 and rs64227627, mean percent density was significantly higher among women with at least one rare allele than among women homozygous for the common allele (*P*=0.01 and *P*=0.03, respectively). For rs11574780, mean percent density was significantly higher among women homozygous for the common allele (*P*=0.03). Two other SNPs, rs1386821 and rs652284 located in *IL-6R*, were of borderline significance (*P*=0.05) and (*P*=0.06).

When poor quality mammograms (N=23) were excluded from the analysis, results did not change significantly; rs6427627 (*P*=0.01), rs11265608 (*P*=0.02) and rs11574780 (*P*=.01), were all still significant. Linkage disequilibrium (LD) analysis was conducted to ensure none of the significant SNPs in *IL6-R* were in high LD. R^2 was greater than .80 for only rs2228145 and rs6684439 (R^2=.86) in *IL-6R* neither of which were significantly associated with mammographic density.

Table 13:. Selected characteristics of the study population

	All study participants N_{total}=369 N(%)
Age (in years):	
- younger than 50	3 (0.8)
- 50-59	164 (44.4)
- 60-69	128 (34.7)
- 70 or older	74 (20.1)
Body mass index (in kg/m^2):	
- normal (less than 25.0)	124 (33.6)
- overweight (25.0-<30.0)	130 (35.2)
- obese (30.0 or more)	115 (31.2)
Smoking:	
- never	211 (57.2)
- former	138 (37.4)
- current	20 (5.4)
Current NSAID use	182 (49.3)
First-degree relative with breast cancer*	55 (15.0)
Previous biopsy	55 (14.9)
Older than 12 years of age at menarche	184 (49.9)
Ever been pregnant	308 (83.5)
Age at first pregnancy lasting ≥6 months (in years):	
- younger than 20	28 (7.6)
- 20-24	131 (35.5)
- 25-29	88 (23.9)
- 30 or older	50 (13.6)
- never pregnant/no pregnancies ≥6 months	72 (19.5)
Younger than 50 years of age at menopause**	147 (40.6)
Type of menopause***:	
- natural menopause	263 (74.1)
- hysterectomy without oophorectomy	41 (11.6)
- hysterectomy with uni- or bilateral oophorectomy	51 (14.4)
Postmenopausal hormone therapy use:	
- never	133 (36.0)
- former	186 (50.4)
- current (within previous 3 months)	50 (13.6)

* 3 missing,** 7 missing,*** 14 missing

Table 14: Variation in cytokine genes and percent mammographic density

SNP ***IL-6* (Chr7)**	Chr Position	Genotype	Percent MD unadjusted LS Mean	Percent MD* adjusted LS Mean	P_{GLM}**	P_{TREND}***
rs1800795†	22733170	G/G (N=132)	25.87	24.93	0.22	0.56
		G/C (N=165) or C/C (N=56)	26.65	27.23		
rs2069827	22731981	G/G (N=298)	27.09	26.81	0.33	0.4
		G/T (N=50) or T/T (N=4)	22.90	24.33		
rs7801617	22724607	G/G (N=268)	26.88	26.74	0.43	0.43
		G/A (N=81) or A/A (N=10)	24.70	25.09		
rs2069840	22735097	C/C (N=163)	26.30	26.85	0.64	0.65
		G/C (N=160) or G/G	26.45	25.98		
rs2069861	22738179	C/C (N=295)	26.94	26.19	0.71	0.83
		C/T (N=61) or T/T (N=3)	23.70	27.08		
rs12700386	22729534	C/C (N=245)	26.14	26.56	0.75	0.91
		G/C (N=100) or G/G	26.86	25.93		
rs2069837	22734552	A/A (N=305)	26.04	26.43	0.77	0.66
		G/A (N=51) or G/G (N=3)	27.89	25.68		
rs7805828	22725087	G/G (N=140)	26.09	26.73	0.83	0.80
		A/G (N=160) or A/A	26.75	26.33		
rs2069860	22737563	A/A (N=354)	26.32	26.33	0.89	0.89
		A/T (N=5)	25.98	25.28		
***IL-6R* (Chr 1)**						
rs6427627	152625803	T/T (N=138)	23.65	23.57	**0.01**	0.23
		C/T (N=170) or C/C(N=47)	28.34	28.39		
rs11265608	152630764	G/G (N=299)	25.25	25.45	**0.03**	**0.02**
		A/G (N=56) or A/A (N=4)	31.96	30.88		
rs1386821	152648673	A/A (N=230)	25.20	25.03	**0.05**	0.16
		C/A (N=113) or C/C (N=16)	28.46	28.78		
rs4240872	152702819	T/T (N=215)	28.08	27.56	0.11	0.24
		C/T (N=127) or C/C (N=17)	23.86	24.58		
rs10159236	152698029	C/C (N=234)	25.21	25.58	0.25	0.24
		C/A (N=105) or A/A	28.60	27.84		
rs4072391	152705504	C/C (N=242)	27.16	26.88	0.38	0.58
		C/T (N=104) or T/T (N=13)	24.63	25.18		
rs2228145	152693594	A/A (N=117)	27.23	27.43	0.4	0.34
		C/A (N=187) or C/C (N=48)	25.89	25.8		
rs11265622	152718044	G/G (N=143)	26.89	27.11	0.47	0.79
		A/G (N=156) or A/A	25.94	25.79		
rs2054855	152637562	C/C(N=256)	26.29	26.05	0.64	0.76
		C/T (N=92) or T/T (N=11)	26.40	27.00		
rs6684439	152662463	C/C (N=124)	26.62	26.96	0.77	0.51
		C/T (N=178) or T/T (N=47)	26.59	26.41		
rs4845618	152666639	T/T (N=125)	27.45	26.09	0.85	0.70
		G/T (N=178) or G/G	25.72	26.44		
rs4601580	152661041	A/A (N=106)	26.64	26.23	0.98	0.87
		A/T (N=189) or T/T (N=62)	26.10	26.28		

Table 14 continued:

SNP	Chr Position	Genotype	Percent MD unadjusted LS Mean	Percent MD* adjusted LS Mean	P_{GLM}**	P_{TREND}***
***IL-6ST* (chr 5)**						
rs11574780	55279795	A/A (N=311)	27.522	27.09	**0.03**	**0.03**
		A/G (N=34)	17.38	20.69		
rs10940495	55298417	A/A (N=189)	27.333	27.67	0.13	0.31
		G/A (N=150) or G/G	25.272	24.92		
rs1063560	55307842	C/C (N=341)	26.69	26.64	0.14	0.14
		C/G (N=15)	19.463	20.35		
rs10471417	55256636	A/A (N=165)	24.317	25.53	0.4	0.58
		C/A (N=147) or C/C	28.2	27.09		
rs6870870	55330085	C/C (N=136)	26.709	27.19	0.51	0.41
		C/A (N=168) or A/A	26.225	25.93		
rs2228043	55279795	C/C (N=289)	26.163	26.34	0.98	0.83
		C/G (N=63) or G/G (N=5)	27.183	26.41		
rs1900173	55275763	T/T (N=309)	26.241	26.38	1	0.77
		T/A (N=37) or A/A (N=2)	27.455	26.36		
***TNFRSF1A* (chr 12)**						
rs4149584	6312904	G/G(N=338)	25.984	26.13	0.27	0.27
		G/A (N=18)	33.98	30.95		
rs4149577	6317783	T/T (N=94)	24.806	25.31	0.49	0.97
		T/C (N=179) or C/C (N=83)	26.932	26.74		
rs4149579	6317618	G/G (N=313)	25.805	26.16	0.65	0.65
		G/A (N=45) or A/A (N=1)	29.936	27.4		
rs2228576	6327323	G/G (N=171)	25.769	26.85	0.70	0.97
		G/A (N=162) or A/A	27.218	26.14		
rs4149570	6321851	G/G (N=131)	24.966	26.06	0.79	0.80
		G/T (N=167) or T/T (N=52)	27.241	26.57		
rs11064145	6325359	T/T (N=103)	27.603	26.03	0.83	0.85
		G/T (N=180) or G/G	25.839	26.48		
rs4149578	6317698	G/G (N=300)	26.078	26.31	0.83	0.65
		G/A (N=51) or A/A (N=4)	28.172	26.87		
***TNFRSF1B* (chr 1)**						
rs652284	12141399	T/T (N=99)	23.12	23.66	0.06	0.32
		T/C (N=182) or C/C (N=78)	27.631	27.41		
rs1201157	12171618	C/C (N=139)	27.427	27.68	0.25	0.50
		C/T (N=165) or T/T (N=52)	25.691	25.54		
rs5746016	12173566	C/C (N=318)	26.394	26.08	0.39	0.38
		T/C (N=34) or T/T (N=1)	25.87	28.78		
rs590977	12177947	A/A (N=238)	27.192	26.89	0.42	0.44
		C/A (N=115) or C/C	24.866	25.37		
rs5746001	12171090	G/G (N=236)	27.12	26.80	0.50	0.49
		G/A (N=105) or A/A	24.903	25.51		
rs653667	12174395	A/A (N=88)	26.586	27.18	0.62	0.31
		C/A (N=207) or C/C	26.334	26.14		
rs683240	12172416	T/T (N=189)	27.768	26.66	0.65	0.65
		C/T (N=147) or C/C (N=22)	24.657	25.85		
rs1061622	12175542	T/T (N=193)	27.587	26.66	0.78	0.73
		G/T (N=140) or G/G	25.076	26.15		
rs816060	12142497	T/T (N=102)	28.123	26.60	0.80	0.32
		C/T (N=178) or C/C (N=77)	25.492	26.08		
***TNF-α* (chr 6)**						
rs1800629†	31651010	G/G (N=278)	26.812	26.87	0.28	0.31
		A/G (N=77) or A/A (N=4)	24.737	24.56		

Legend Table 14:
* LS Mean adjusted for age, BMI, hormone therapy use (never, past, current), NSAID use, pregnancy for at least 6 months and previous biopsy.
** Proc GLM conducted in SAS 9.2 adjusted for BMI, hormone therapy use , NSAID use, pregnancy for at least 6 months and prior breast biopsy.
***Regression conducted in Plink 1.06 for effect of each extra minor allele adjusted for BMI, hormone therapy use, NSAID use, pregnancy for at least 6 months and previous biopsy
┼ Functional SNP

KEY RESEARCH ACCOMPLISHMENTS

- We found that circulating levels of IL-6, TNF-α, and CRP were not independently associated with dense breast area, nondense breast area or percent density among women with benign breast disease or among women with a negative screening mammogram in our study population. We did observe statistically significant, positive associations between these cytokines and nondense breast area and negative associations with percent density in age-adjusted analyses, though further adjustment for BMI caused these associations to be attenuated and non-significant among both groups. Adjustment for additional covariates did not affect these estimates further.

- In a study population consisting of 376 postmenopausal MAMS participants without breast cancer who did not use hormone therapy at the time of enrollment we found that circulating levels of sTNFR1 and sTNFR2 were not independently associated with percent density, dense breast area or nondense breast area. We did observe statistically significant, negative associations between sTNFR levels and percent density and positive associations with nondense breast area in age-adjusted analyses. However, as above, further adjustment for BMI caused these associations to be attenuated and non-significant. While not a primary aim, recent NSAID use reported at blood collection was associated with lower percent mammographic density.

- Using genotype information on 45 SNPs located in or near *IL6*, *IL6R*, *IL6ST*, *TNF-α* , *TNFRSF1A*, and *TNFRSF1B* from.369 healthy Caucasian postmenopausal MAMS participants we found that two common SNPs in *IL6R* and one in *IL6-ST* were statistically significantly associated with percent density.

REPORTABLE OUTCOMES

Manuscript: Reeves KW, Weissfeld JL, Modugno F, and Diergaarde B. Circulating levels of inflammatory markers and mammographic density among postmenopausal women. *Breast Cancer Res Treat*. 2010 Nov 11. [Epub ahead of print] PMID: 21069450.

Poster/abstract: Brand, H., Weissfeld, J.L., and Diergaarde, B. Common variation in inflammation-related genes and mammographic density in postmenopausal women. Poster

presented at the *American Society of Preventive Oncology 34th Annual Meeting*, Bethesda, MD, March 2010.

Abstract: Reeves KW, Weissfeld JL, Modugno F, and Diergaarde B. Inflammatory markers and mammographic density among postmenopausal women. 2011 *Era of Hope*, Orlando, Florida, August 2011.

CONCLUSION

- **Cytokine levels and mammographic density**

  IL-6, TNF-α, CRP, sTNFR1 and sTNFR2 plasma levels were not independently associated with dense breast area, nondense breast area or percent density in our study population, which suggests that these circulating cytokines do not impact breast carcinogenesis through independent effects on mammographic density.

  We had originally hypothesized that greater breast density would be associated with higher circulating levels of TNF-α and IL-6, and lower levels of sTNFR1 and sTNFR2. Instead, we observed that the levels of these 4 cytokines were all positively associated with nondense breast area and inversely with percent density in age-adjusted analyses. Additional adjustment with BMI caused all these associations to be attenuated and non-significant.

  Despite our findings, IL-6, TNF-α, CRP, sTNFR1 and sTNFR2 levels may play an important role in breast carcinogenesis, but it is difficult to separate the effects of these cytokines and BMI/obesity when evaluating their impact on mammographic density. BMI is negatively associated with percent density [13-15], and positively associated with nondense breast area [13, 16] and IL-6, TNF-α, CRP, sTNFR1 and sTNFR2 [17-19]; these associations were apparent in our study population as well. Obesity is characterized by the infiltration of macrophages in adipose tissue, and these macrophages are an important source of TNF-α and IL-6 [20, 21]. Smaller quantities of TNF-α and IL-6 are produced by preadipocytes and adipocytes [22].

  If BMI and circulating levels of IL-6, TNF-α, CRP, sTNFR1 and sTNFR2 are not on the same causal pathway, then our adjustment for BMI is both necessary and appropriate; the conclusion of our results would be that there is truly no independent relationship between these cytokines and nondense breast area and percent density. Alternatively, if BMI and these cytokines affect nondense breast area and percent density through a shared causal pathway, then adjustment for body mass index would not be appropriate. In this case we would have to conclude that all evaluated factors are positively associated with nondense breast area and negatively associated with percent density, as indicated in our age-adjusted regressions. Future research will be required to determine whether or not BMI and these cytokines are on the same causal pathway for mammographic density and/or breast cancer. One way to do this would be to look at markers of obesity such as adiponectin and leptin and their relationship with mammographic density and cytokine levels.
- **Variation in cytokine genes and mammographic density**

Polymorphisms in *IL6*, *TNF-α* and the genes that code for their receptors may alter exposure to estrogens and so affect mammographic density. In line with this, our preliminary results suggest that common variation in *IL6R* and *IL6ST* is associated with percent density in healthy Caucasian postmenopausal women. Identification of the genes (and within the genes the functional polymorphisms) that affect breast density will likely provide further insights into the biology of the breast and may identify potential targets for breast cancer (chemo)prevention.

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APPENDICES

- Manuscript: Reeves KW, Weissfeld JL, Modugno F, and Diergaarde B. Circulating levels of inflammatory markers and mammographic density among postmenopausal women. *Breast Cancer Res Treat*. 2010 Nov 11. [Epub ahead of print] PMID: 21069450.

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EPIDEMIOLOGY

Circulating levels of inflammatory markers and mammographic density among postmenopausal women

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Abstract Mammographic density is strongly associated with breast cancer risk. Inflammation is involved in breast carcinogenesis, perhaps through effects on mammographic density. We evaluated associations between inflammatory markers interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and C-reactive protein (CRP) and mammographic density among postmenopausal women. Plasma IL-6, TNF-α, and CRP levels were measured in 145 women with benign breast disease (benign controls) and 397 women with a negative screening mammogram (well controls) enrolled in the Mammograms and Masses Study. Associations between the inflammatory markers and mammographic density were evaluated separately for benign and well controls through correlation analyses and linear regressions. Age-adjusted mean CRP levels were higher among benign controls (2.07 μg/ml) compared to well controls (1.63 μg/ml; $P = 0.02$), while IL-6 and TNF-α levels were similar between groups. Using linear regression, IL-6, TNF-α, and CRP were not statistically significantly associated with dense breast area within either group. Statistically significant positive associations were observed between all three markers and nondense breast area in both groups; statistically significant negative associations were observed between IL-6 and percent density among benign controls, and between all three markers and percent density among well controls. These associations were all attenuated and non-significant upon adjustment for body mass index. IL-6, TNF-α, and CRP levels were not independently associated with dense breast area, nondense breast area, or percent density in this study population. Our results suggest that these inflammatory factors do not impact breast carcinogenesis through independent effects on mammographic density.

Keywords Interleukin-6 (IL-6) · Tumor necrosis factor-α (TNF-α) · C-reactive protein (CRP) · Mammographic density · Postmenopausal

Abbreviations

ANOVA	Analysis of variance
BMI	Body mass index
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
IL-6	Interleukin-6
LCBR	Laboratory for Biochemsitry Research
MAMS	Mammograms and Masses Study
NSAID	Non-steroidal anti-inflammatory drug
TNF-α	Tumor necrosis factor alpha

Introduction

Mammographic density is positively associated with breast cancer risk, and it may represent an associated phenotype

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for this disease [1]. Mammographic density refers to the amount of connective and epithelial tissue present in the breast relative to fat as viewed on a mammogram [2, 3]. Two common measurements of mammographic density are dense breast area and percent density. Percent density is the more frequently used measure, yet dense breast area also is strongly related to breast cancer risk [4–6]. The heritability of percent density is estimated to be 63% [7]. Thus, more than one-third of the variability of breast density is influenced by other, potentially modifiable, factors. Indeed, studies have demonstrated that mammographic density changes in response to factors such as use [8, 9] or cessation [10] of hormone therapy. Mammographic density also changes during the menstrual cycle [11].

Estrogen plays a critical role in breast carcinogenesis, and exposure to both endogenous [12, 13] and exogenous [14, 15] estrogens is positively associated with breast cancer risk. In recent years evidence has emerged that breast cancer etiology may also have an inflammatory component. Inflammatory factors might influence breast cancer risk through their effects on the estrogen pathway. For example, breast cancer risk is approximately 20% lower among women who regularly use non-steroidal anti-inflammatory drugs (NSAIDs) [16–19]. Aspirin use decreases risk of progression to breast cancer among women with benign breast disease [20]. NSAIDs block cyclooxygenase-2 (COX-2), an enzyme that converts arachidonic acid into prostaglandins, which in turn trigger increased estrogen formation in adipose tissue [21]. The inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) increase the production of aromatase, the enzyme responsible for estrogen production in adipose tissue via conversion of androstenedione to estrone [22, 23]. This action of IL-6 and TNF-α is especially important in postmenopausal women, as estrone is the primary form of estrogen produced after the menopause [24]. Levels of the acute-phase inflammatory marker C-reactive protein (CRP) are decreased when COX-2 action is inhibited [25]. Thus, IL-6, TNF-α, and CRP may provide a link between the inflammatory and estrogen pathways thought to be important to the development of breast cancer.

Studies examining circulating levels of IL-6, TNF-α, or CRP in relation to breast cancer risk have provided inconsistent results. Some studies report no association between IL-6 and risk of breast cancer [26, 27] or cytologic atypia [28], while another observed elevated IL-6 levels among breast cancer cases with insulin resistance [29]. Five studies reported no significant association between TNF-α and breast cancer [26, 29–32], while one found decreased production of TNF-α from T lymphocytes in breast cancer patients [33] and another observed increased levels of TNF-α among breast cancer cases [34]. Most studies of CRP and breast cancer risk found no statistically significant association [26, 27, 35–37], though one reported a significant positive association [38]. An additional study reported a weak, positive association between CRP in nipple aspirate fluid and Gail score, an indicator of breast cancer risk [39]. Many of the published studies on IL-6, TNF-α, and CRP and breast cancer risk, however, are affected by numerous biases and limitations, including small sample sizes, inadequate description of laboratory procedures and quality control, and failure to control for potential confounders. Additionally, the relationship between these inflammatory factors and benign breast disease has not been described. Inflammation is an extremely complex process, and the exact mechanisms by which inflammation relates to the occurrence of breast cancer are not yet known. It may be that inflammatory factors impact breast carcinogenesis through a pathway which includes mammographic density, yet this has not been explored in published reports.

We evaluated associations between IL-6, TNF-α, and CRP levels and measures of mammographic density (dense breast area, nondense breast area, and percent density) in a large sample of postmenopausal women on whom extensive covariate data were available. To our knowledge, no previous studies have investigated associations between IL-6, TNF-α or CRP, and mammographic density.

Materials and methods

Study population

We conducted a cross-sectional investigation using controls participating in the Mammograms and Masses Study (MAMS), a case–control study on hormones and mammographic density. Details of MAMS have been described elsewhere [40, 41]. Briefly, women were eligible for MAMS if they were 18 years or older and were visiting Magee-Womens Hospital (Pittsburgh, PA) or a Magee Womancare Center in the greater Pittsburgh area for one of the following: (a) a breast biopsy, (b) an initial surgical consultation after breast cancer diagnosis, or (c) a routine screening mammogram. Women were excluded if they reported a prior cancer history other than non-melanoma skin cancer, drank more than five alcoholic beverages per day, or weighed less than 110 lb or more than 300 lb. Recruitment took place from September 2001 to May 2005. Pathology reports were used to determine disease status (benign breast disease, in situ breast cancer, invasive breast cancer) for those undergoing a breast biopsy and/or surgery. The MAMS study population consists in total of 1,133 women: 264 women with in situ or invasive breast cancer (cases), 313 women with benign breast disease (benign controls), and 556 women with a negative screening

mammogram (well controls). The University of Pittsburgh Institutional Review Board reviewed and approved the study protocol, and all study participants provided written informed consent.

Both benign and well controls were included in the present study ($N_{total} = 869$). For the current analyses, we subsequently excluded all women who were not postmenopausal ($N = 222$), had no available mammogram data ($N = 53$), did not complete the questionnaire ($N = 26$), reported a prior history of cancer after enrollment into MAMS ($N = 9$), had no available plasma sample ($N = 8$) or whose blood draw was more than 180 days from their mammogram date ($N = 9$), leaving a final total of 145 benign controls and 397 well controls. We excluded premenopausal women because fluctuating hormone levels during the menstrual cycle can affect cytokine levels in premenopausal women and specific information on day of the menstrual cycle at time of mammogram was not available.

Data collection

Information on medical history, reproductive history, lifestyle factors such as smoking status and alcohol intake, demographic characteristics, medication use, and family history of breast cancer was collected using a self-administered questionnaire. Women were assumed to be postmenopausal if they had no periods in the year before enrollment, had ever used hormone therapy, had had a bilateral oophorectomy, or were 60 years or older at enrollment. Women who reported a hysterectomy without bilateral oophorectomy were considered to be postmenopausal if they had ever used hormone therapy or were 50 years or older at hysterectomy. Age at menopause was set to age at which menstrual periods ended, age at a bilateral oophorectomy, or age of first use of hormone therapy, whichever came first. For women who had a hysterectomy without bilateral oophorectomy, age at menopause was set to age at which they first used hormone therapy or first had menopausal symptoms, whichever came first. If neither occurred and age at hysterectomy was 50 years or older, then age at menopause was age at hysterectomy. Height and weight were measured by a research nurse using a stadiometer and a standard balance beam scale while participants wore light clothing and no shoes. Body mass index (BMI) was computed as weight (in kg) divided by height squared (in meters). The summary variable 'current NSAID use' was created as described previously [41]. A non-fasting, 40 ml sample of peripheral blood was collected from the study participants at enrollment. All samples were processed immediately at the Magee-Womens Hospital Clinical Research Center and stored at $<-70°C$. Blood samples were taken an average of 34 days (SD 29 days) after the mammogram. The majority (58.5%) of blood samples was collected within 31 days of the mammogram; 94% were collected within 90 days of the mammogram. The time interval from mammogram to blood collection did not differ significantly between benign and well controls ($P = 0.47$).

Laboratory assays

Circulating levels of IL-6, TNF-α, and CRP were measured in frozen stored EDTA plasma samples by the Laboratory for Clinical Biochemistry Research (LCBR) at the University of Vermont (Colchester, VT). Samples were shipped to the LCBR packed in dry ice using overnight courier service. Investigators at the LCBR were blinded to the identity, demographic and risk factor characteristics, and mammographic density status of the samples. To evaluate assay reproducibility, 36 masked, duplicate samples (6.6% of total study samples) were randomly distributed throughout the batch of samples. Plasma IL-6 levels were measured using a high sensitivity enzyme-linked immunosorbent assay (ELISA; Human IL-6 Quantikine® HS, HS600B) from R&D Systems (Minneapolis, MN). The detectable limit for IL-6 was 0.10 pg/ml, and the average coefficient of variation (CV) was 16.0%. TNF-α levels were measured by a singleplex immunoassay using Luminex technology (Human Cytokine LINCOplex Kit Singleplex TNF-α, HCYTO-60K-1TNFA; Linco Research, Inc., St. Charles, MO). This assay can measure TNF-α concentrations $\leq$3.2 pg/ml, and the average CV was 10.8%. CRP levels were measured using the BNII nephelometer from Dade Behring utilizing a particle-enhanced immunonepholometric assay. This assay has a detection limit of 0.16 μg/ml, and the average CV was 9.6%.

Mammographic density measurements

Copies of participants' most recent screening mammograms were obtained with their permission. The assessment of mammographic measures has been described in detail elsewhere [40]. Briefly, one expert reader read all mammograms, which were copies of the original films. This reader was masked to the identity, status (benign control, well control), and demographic and risk factor characteristics of the subject. Total breast area and all dense regions were measured using a compensating polar planimeter (LASICO) on the craniocaudal view with the side of breast (right or left) randomly chosen for each participant. Dense breast area is the sum of all dense regions; nondense breast area was calculated by subtracting dense breast area from total breast area; percent density was calculated by dividing dense breast area by total breast area and multiplying that by 100. A subjective measure of film quality was also reported (excellent, good, fair, poor, very poor, extremely poor) by the expert reader. In a separate reproducibility

study using mammograms from 28 MAMS participants, intraclass correlation coefficients for dense breast area, total breast area and percent density were $\rho = 0.86$, $\rho = 0.99$, and $\rho = 0.89$, respectively [40].

Statistical analysis

All analyses were performed separately for benign controls and well controls. Differences between benign and well controls were assessed using chi-square tests for categorical variables and *t*-tests or analysis of variance (ANOVA) for continuous variables. The normality of the distribution of circulating IL-6, TNF-α, and CRP levels, and dense breast area, nondense breast area, and percent density was assessed graphically using quantile–quantile plots. To improve normality, natural log transformations were applied to the inflammatory markers and square root transformations were applied to the mammographic density measures. Pearson's correlation coefficient was used to examine the correlation between cytokine levels and mammographic measures; Fisher's z transformations were used to test differences between the correlation coefficients. Linear regression was used to further assess the association between each inflammatory marker and mammographic density. The assumptions needed for linear regression were met. Unadjusted, age-adjusted, age- and BMI-adjusted, and multivariable-adjusted regression models were run for each combination of inflammatory marker and mammographic density measure. The multivariable model included covariates found to be associated with mammographic density and/or breast cancer in previous studies: age (continuous), BMI (<25 kg/m^2, 25 to <30 kg/m^2, ≥ 30 kg/m^2), race (white, other), smoking (never, former, current), current NSAID use (nonuser, user), first-degree relative with breast cancer (no, yes), age at menarche (≤ 12, >12), age at menopause (<50, ≥ 50), type of menopause (natural, hysterectomy without oophorectomy, hysterectomy with uni- or bilateral oophorectomy), prior breast biopsy (no, yes), ever been pregnant (no, yes), and postmenopausal hormone therapy use status (never, former, current). We subsequently repeated the regressions stratified by BMI, current NSAID use, and time between blood draw and mammogram, and separately among participants with high quality mammograms. *P* values <0.05 were considered statistically significant. Analyses were performed using Stata (version 10.0; Stata Corporation, College Station, TX) and SAS (version 9.2; SAS Institute Inc., Cary, NC) software.

Results

The study population consisted of two groups: 145 women with benign breast disease (benign controls) and 397 women with a negative screening mammogram (well controls). Characteristics of the study population by control status are presented in Table 1. Mean age among benign controls was statistically significantly lower than among well controls. Additionally, benign controls reported having had a prior breast biopsy, to be older than 12 years of age at menarche, to have gone through menopause before age 50, and to be current users of postmenopausal hormone therapy statistically significantly more often than well controls. Current use of NSAIDs was significantly more common among the well controls.

The distributions of the inflammatory markers and the mammographic density measures by control status are shown in Table 2. The age-adjusted geometric mean of CRP was statistically significantly higher among benign controls (2.07 μg/ml) than among well controls (1.63 μg/ml; $P = 0.02$). No significant differences between benign controls and well controls were observed for IL-6 and TNF-α. Regarding the mammographic density measures, age-adjusted mean dense breast area (42.8 vs. 36.1 cm^2; $P = 0.02$) and age-adjusted mean percent density (31.2 vs. 26.0%; $P = 0.01$) were both statistically significantly higher among benign controls than among well controls. Age-adjusted mean nondense breast area did not differ significantly between the two groups (97.8 vs. 108.1 cm^2; $P = 0.13$).

No significant correlations were observed between circulating IL-6, TNF-α, and CRP levels and dense breast area among benign or well controls (Table 3). However, all three cytokines were statistically significantly, positively correlated with nondense breast area in both control groups (benign controls: IL-6: $\rho = 0.32$, $P < 0.001$; TNF-α: $\rho = 0.22$, $P < 0.001$; CRP: $\rho = 0.25$, $P = 0.003$; well controls: IL-6: $\rho = 0.30$, $P < 0.001$; TNF-α: $\rho = 0.26$, $P < 0.001$; CRP: $\rho = 0.36$, $P < 0.001$), and statistically significantly, negatively correlated with percent density among well controls (IL-6: $\rho = -0.20$, $P < 0.001$; TNF-α: $\rho = -0.18$, $P < 0.001$; CRP: $\rho = -0.23$, $P < 0.001$). Among benign controls the correlation with percent density was statistically significant for IL-6 ($\rho = -0.21$, $P = 0.01$) and borderline significant for CRP ($\rho = -0.16$, $P = 0.05$). TNF-α levels were also negatively correlated with percent density among benign controls, yet this association was not statistically significant. No statistically significant differences in correlation coefficients for IL-6, TNF-α, and CRP were observed between benign controls and well controls (Table 3).

Results from the age-adjusted, age- and BMI-adjusted, and multivariable-adjusted linear regression models are presented in Table 4. For both benign controls and well controls, no statistically significant associations were observed between levels of IL-6, TNF-α, and CRP and dense breast area in any of the models but all three

Table 1 Selected characteristics of the study population by control status ($N_{total} = 542$)

	Benign controls ($N = 145$)[a] *N* (%)	Well controls ($N = 397$)[a] *N* (%)	P[b]
Age (years; mean ± SD)	58.3 ± 7.4	62.0 ± 8.1	<0.001
Age (years)			<0.001
Younger than 50	12 (8.3)	4 (1.0)	
50–59	68 (46.9)	180 (45.3)	
60–69	55 (37.9)	135 (34.0)	
70 or older	10 (6.9)	78 (19.7)	
Race: White	136 (93.8)	373 (94.0)	0.94
Body mass index (kg/m^2; mean ± SD)	27.9 ± 5.9	28.3 ± 6.0	0.57
Body mass index (kg/m^2)			0.46
Normal (less than 25.0)	44 (30.6)	131 (33.0)	
Overweight (25.0 to <30.0)	58 (40.3)	137 (34.5)	
Obese (30.0 or more)	42 (29.2)	129 (32.5)	
Smoking status			0.07
Never	81 (53.6)	227 (57.2)	
Former	45 (31.3)	144 (36.3)	
Current	18 (12.5)	26 (6.6)	
Prior breast biopsy	60 (41.7)	57 (14.4)	<0.001
First-degree relative with breast cancer	18 (12.5)	56 (14.2)	0.61
Age at menarche (years)			0.04
12 or younger	58 (40.0)	197 (49.8)	
Older than 12	87 (60.0)	199 (50.3)	
Ever been pregnant	121 (83.5)	332 (83.6)	0.96
Age at first pregnancy lasting ≥6 months			0.36
Never pregnant/no pregnancies ≥6 months	32 (22.2)	80 (20.2)	
Younger than 20	18 (12.5)	35 (8.8)	
20–24	52 (36.1)	143 (36.0)	
25–29	27 (18.8)	89 (22.4)	
30 or older	15 (10.4)	50 (12.6)	
Age at menopause (years)			<0.001
Younger than 50	85 (59.9)	164 (42.2)	
50 or older	57 (40.1)	225 (57.8)	
Type of menopause			0.13
Natural menopause	90 (65.7)	275 (72.4)	
Hysterectomy without oophorectomy	16 (11.7)	48 (12.6)	
Hysterectomy with uni- or bilateral oophorectomy	31 (22.6)	57 (15.0)	
Postmenopausal hormone therapy use			<0.001
Never	27 (18.8)	140 (35.3)	
Former	43 (29.9)	203 (51.1)	
Current (within previous 3 months)	74 (51.4)	54 (13.6)	
Current NSAID use	42 (34.4)	194 (49.2)	0.004

[a] The numbers do not always add up to the total number of benign and well controls due to missing information

[b] Chi-square tests for categorical variables and *t*-tests for continuous variables

inflammatory markers were statistically significantly associated with nondense breast area in the age-only adjusted model (benign controls: IL-6: $\beta = 1.44, P < 0.001$; TNF-α: $\beta = 1.46$, $P = 0.007$; CRP: $\beta = 0.70$, $P = 0.003$; well controls: IL-6: $\beta = 1.49$, $P < 0.001$; TNF-α: $\beta = 1.78$, $P < 0.001$; CRP: $\beta = 1.18$, $P < 0.001$). The associations with nondense breast area became non-significant after additional adjustment for BMI and other variables. Among benign controls, IL-6 was statistically significantly associated with percent density in the age-only adjusted model ($\beta = -0.55$, $P = 0.02$). This association became non-significant upon further adjustment for BMI, and remained non-significant upon adjustment for additional covariates. No significant associations were observed for TNF-α and

Table 2 Distribution of inflammatory markers and mammographic density measures by control status

	Benign controls				Well controls				P^b
	N	Mean (SD)	Median	Age-adjusted transformed mean[a]	*N*	Mean (SD)	Median	Age-adjusted transformed mean[a]	
Inflammatory markers[c]									
IL-6 (pg/ml)	145	2.67 (2.72)	1.97	2.12	397	2.89 (2.91)	1.98	2.17	0.71
TNF-α (pg/ml)	145	3.00 (1.60)	2.59	2.67	394	2.99 (1.83)	2.68	2.63	0.70
CRP (μg/ml)	142	4.16 (8.57)	2.17	2.07	381	2.92 (4.17)	1.47	1.63	0.02
Mammographic density measures									
Dense breast area (cm^2)	145	48.0 (30.6)	44.6	42.8	397	40.9 (26.6)	36.7	36.1	0.02
Nondense breast area (cm^2)	145	106.0 (71.9)	90.5	97.8	397	120.7 (76.3)	100.1	108.1	0.13
Percent density (%)	145	35.2 (18.8)	34.2	31.2	397	29.6 (19.4)	27.5	26.0	0.01

[a] Transformed mean is geometric mean for the inflammatory markers. For the mammographic density variables, the transformed mean is a mean calculated on the square root scale that was subsequently transformed back to the original scale

[b] *P* values from ANOVA, comparing distributions among benign controls to well controls using natural log transformations of the inflammatory markers and square root transformations of the mammographic density variables with adjustment for age

[c] TNF-α levels could not be measured for three well controls; CRP levels could not be measured for three benign controls and 16 well controls

Table 3 Correlations between inflammatory markers and mammographic density measures by control status[a]

	Benign controls			Well controls			P^b
	N	ρ	*P*	*N*	ρ	*P*	
Dense breast area							
IL-6	145	−0.03	0.72	397	−0.06	0.24	0.77
TNF-α	145	0.04	0.64	394	−0.01	0.78	0.59
CRP	142	−0.01	0.95	381	−0.03	0.60	0.83
Nondense breast area							
IL-6	145	0.32	<0.001	397	0.30	<0.001	0.90
TNF-α	145	0.22	<0.001	394	0.26	<0.001	0.65
CRP	142	0.25	0.003	381	0.36	<0.001	0.22
Percent density							
IL-6	145	−0.21	0.01	397	−0.20	<0.001	0.90
TNF-α	145	−0.11	0.19	394	−0.18	<0.001	0.44
CRP	142	−0.16	0.05	381	−0.23	<0.001	0.50

[a] Calculated using Pearson's correlation coefficient with natural log transformations of the inflammatory markers and square root transformations of the mammographic density variables

[b] *P* values for comparison of correlation coefficients between benign controls and well controls

CRP levels with percent density among women with benign breast disease in any model. Among well controls, all three inflammatory markers were statistically significantly associated with percent density in the age-only adjusted model (IL-6: $\beta = -0.54$, $P < 0.001$; TNF-α: $\beta = -0.71$, $P < 0.001$; CRP: $\beta = -0.43$, $P < 0.001$). These associations became non-significant after additional adjustment for BMI and other variables.

Subsequently, regressions were repeated stratified by BMI, current NSAID use, and time between blood draw and mammogram; results were generally similar to those observed overall (data not shown). Additionally, results were similar to those observed in the total populations of benign controls and well controls when regressions were restricted to women with mammograms of good or excellent film quality (data not shown).

Discussion

Plasma levels of IL-6, TNF-α, and CRP were not independently associated with dense breast area, nondense breast area, or percent density among women with benign breast disease or among women with a negative screening mammogram in our study population. We did observe statistically significant, positive associations between these inflammatory factors and nondense breast area and negative associations with percent density in age-adjusted analyses, though further adjustment for BMI caused these associations to be attenuated and non-significant among both groups. Adjustment for additional covariates did not affect these estimates further.

It is of interest that CRP levels were statistically significantly elevated among women with benign breast disease compared to the well controls. This may reflect true effects of benign breast disease on inflammation, or vice versa, or it may relate to the lower NSAID use observed among benign versus well controls. Benign controls were recruited and gave a blood sample at the time of their breast biopsy, and their less frequent NSAID use may reflect instructions given by their physician to avoid NSAID use prior to the biopsy procedure. Dense breast area and percent density were significantly greater among

Table 4 Results of linear regressions of mammographic density measures on inflammatory markers by control status[a]

	Age-adjusted			Age- and BMI-adjusted			Fully adjusted[b]		
	N	β (SE)	*P*	*N*	β (SE)	*P*	*N*	β (SE)	*P*
Benign controls									
Dense breast area									
IL-6	145	−0.13 (0.28)	0.64	144	−0.07 (0.31)	0.83	111	−0.07 (0.39)	0.85
TNF-α	145	0.18 (0.37)	0.63	144	0.26 (0.39)	0.50	111	0.40 (0.50)	0.43
CRP	142	−0.01 (0.16)	0.95	141	0.001 (0.17)	0.99	109	0.16 (0.21)	0.45
Nondense breast area									
IL-6	145	1.44 (0.39)	<0.001	144	0.23 (0.35)	0.52	111	0.09 (0.45)	0.84
TNF-α	145	1.46 (0.53)	0.007	144	0.50 (0.44)	0.26	111	0.03 (0.58)	0.96
CRP	142	0.70 (0.23)	0.003	141	0.16 (0.19)	0.42	109	0.06 (0.24)	0.82
Percent density									
IL-6	145	−0.55 (0.23)	0.02	144	−0.08 (0.23)	0.74	111	−0.03 (0.30)	0.93
TNF-α	145	−0.42 (0.31)	0.18	144	−0.03 (0.29)	0.92	111	0.19 (0.35)	0.62
CRP	142	−0.26 (0.13)	0.06	141	−0.05 (0.13)	0.69	109	0.06 (0.17)	0.71
Well controls									
Dense breast area									
IL-6	397	−0.16 (0.16)	0.32	397	−0.07 (0.17)	0.69	368	−0.15 (0.18)	0.41
TNF-α	394	−0.04 (0.23)	0.87	394	0.09 (0.24)	0.71	365	0.04 (0.25)	0.87
CRP	381	−0.06 (0.11)	0.57	381	0.01 (0.12)	0.96	353	0.02 (0.13)	0.85
Nondense breast area									
IL-6	397	1.49 (0.24)	<0.001	397	0.39 (0.20)	0.05	368	0.34 (0.22)	0.12
TNF-α	394	1.78 (0.34)	<0.001	394	0.35 (0.28)	0.21	365	0.32 (0.30)	0.29
CRP	381	1.18 (0.16)	<0.001	381	0.27 (0.14)	0.06	353	0.29 (0.16)	0.06
Percent density									
IL-6	397	−0.54 (0.14)	<0.001	397	−0.11 (0.14)	0.43	368	−0.13 (0.15)	0.38
TNF-α	394	−0.71 (0.20)	<0.001	394	−0.16 (0.19)	0.39	365	−0.17 (0.20)	0.41
CRP	381	−0.43 (0.09)	<0.001	381	−0.08 (0.10)	0.44	353	−0.07 (0.10)	0.48

[a] Regressions performed using natural log transformations of the inflammatory markers and square root transformations of the mammographic measures

[b] Adjusted for age, race, BMI, smoking, current NSAID use, first-degree relative with breast cancer, age at menarche, age at menopause, type of menopause, prior breast biopsy, ever been pregnant, and postmenopausal hormone therapy use

benign versus well controls. This finding is in agreement with a prior study documenting strong correlation between dense breast area and percent density and history of atypical hyperplasia or lobular carcinoma in situ [42]. These differences support our decision to consider women with benign breast disease separate from women with negative screening mammograms in our analyses.

Our results indicate that IL-6, TNF-α, and CRP do not independently affect breast cancer risk through a pathway that includes mammographic density. The positive and negative age-adjusted associations that we observed with nondense breast area and percent density, respectively, were attenuated and became non-significant when adjusted for BMI. BMI is negatively associated with percent density [43–45], and BMI is positively associated with nondense area [46] and IL-6, TNF-α, and CRP [26, 47, 48]; these associations were apparent in our study population as well (data not shown). Obesity is characterized by infiltration of macrophages in adipose tissue, and these macrophages are an important source of TNF-α and IL-6 [49, 50]. Smaller quantities of TNF-α and IL-6 are produced by preadipocytes and adipocytes [51]. IL-6, TNF-α, and CRP may play an important role in breast carcinogenesis, but it is difficult to separate the effects of the inflammation markers and BMI when evaluating their influences on percent density.

If BMI and circulating levels of IL-6, TNF-α, and CRP are not on the same causal pathway, then our adjustment for BMI is both necessary and appropriate; the conclusion of our results would be that there is truly no independent relationship between these inflammatory markers and nondense breast area and percent density. Alternatively, if BMI and these inflammatory markers affect percent density

through a shared causal pathway, then adjustment for BMI would not be appropriate. In this case we would have to conclude that IL-6, TNF-α, and CRP are positively associated with nondense breast area and negatively associated with percent density, as indicated in our age-adjusted regressions. Future research will be required to determine whether or not BMI and these inflammatory markers are on the same causal pathway for mammographic density and/or breast cancer.

An additional possibility is that percent density is not an appropriate measure for studying etiologic associations between biomarkers and mammographic density. Percent density represents both the number of cells at risk for breast cancer (dense breast area) and the amount of fat tissue (nondense area) in the breast, which is highly correlated with BMI [43]. Therefore, observed associations between percent density and exposures that are strongly associated with BMI may not indicate direct effects of such exposures on the dense breast tissue [43]. The issues related to BMI and percent density do not appear to be unique to IL-6, TNF-α, and CRP, but rather occur with other BMI-associated exposures as well. As a result, it may be more appropriate to use dense breast area as the preferred measure of mammographic density in etiologic studies [43, 45]. In our study population none of the inflammatory factors investigated were related to dense breast area even in unadjusted analyses.

Previous studies provide inconsistent evidence for IL-6, TNF-α, and CRP in relation to breast carcinogenesis. The majority of studies have found no association, yet some found positive associations with IL-6 [29], TNF-α [34], and CRP [38]. Two of these studies [29, 34] did not adjust for BMI, however, which, as discussed above, may or may not confound the observed associations. Based on our results, it is not clear that IL-6, TNF-α, or CRP is independently associated with mammographic density, an associated phenotype for breast cancer.

Limitations of this study primarily relate to the measurement of the inflammatory factors. IL-6, TNF-α, and CRP were all measured at a single time-point, and therefore may not be representative of a participant's usual levels. Circulating levels of IL-6, TNF-α, and CRP could reflect recent changes in general health or medication use. In particular, current use of NSAIDs could greatly impact circulating levels of these factors. However, we were able to control for current use of NSAIDs in our analysis, and recent studies have demonstrated reasonable within-subject stability of serum IL-6, TNF-α, and CRP levels over 1 year [52] and over repeated monthly measurements [53]. Additionally, the correlation between circulating and breast tissue levels of these factors has not been established. Tissue levels might be more relevant to breast carcinogenesis and might possibly show a different association with mammographic density. Finally, external validity is limited by the racial homogeneity, high socioeconomic status, and overall good health of the study population. Our study is strengthened by our large sample size, use of healthy subjects, and the high reliability of our IL-6, TNF-α, CRP, and mammographic density measurements.

Though inflammatory pathways may be important to breast carcinogenesis, our results suggest that the inflammatory markers IL-6, TNF-α, and CRP do not impact breast carcinogenesis through independent effects on mammographic density. Future research is needed to elucidate the exact mechanisms by which inflammation is related to breast cancer risk.

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- Chris Kline

Statement of Work - Inflammatory Markers and Breast Cancer Risk

Task 1: Obtain Human Subject's Approval from the DOD (Months 1-3) - completed

Task 2: Data and Specimen Identification (Months 1-3) - completed

Task 3: Specimen pulling and shipping (Months 4-6) - completed

Task 4: Isolate DNA (Months 6-12) - completed

Task 5: Perform Cytokine Assays (Months 6-18) - completed

Task 6: Identify SNPs and Haplotype Tagging SNPs (htSNPs) (Months 13-15) - completed

Task 7: Perform Genotyping Assays (Months 16-22) - completed

Task 8: Create Study Database (Months 22-24) - completed

Task 9: Analyze Cytokine Data (Months 25-30) - completed

Task 10: Analyze SNP Data (Months 25-30) - completed

Task 11: Disseminate Results (Months 30-36) - ongoing
We will continue to work on the dissemination of the results.